Further Evidence of the Association of the Diacylglycerol Kinase Kappa (*DGKK*) Gene With Hypospadias

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Purpose: Hypospadias is a common developmental anomaly of the male external genitalia. In previous studies conducted on West European, Californian, and Han Chinese populations the relationship between polymorphic variants of the diacylglycerol kinase kappa (*DGKK*) gene and hypospadias have been reported. The aim was to study the possible associations between polymorphic variants of the *DGKK* gene and hypospadias using an independent sample of the Polish population.

Materials and Methods: Ten single nucleotide polymorphisms in *DGKK*, which were reported to have an impact on the risk of hypospadias in other populations, were genotyped using high-resolution melting curve analysis in a group of 166 boys with isolated anterior (66%) and middle (34%) forms of hypospadias and 285 properly matched controls without congenital anomalies.

Results: Two *DGKK* variants rs11091748 and rs12171755 were associated with increased risk of hypospadias in the Polish population. These results were statistically significant, even after applying the Bonferroni correction for multiple comparisons ($P < .005$). All the tested nucleotide variants were involved in haplotype combinations associated with hypospadias. The global p-values for haplotypes comprising of rs4143304-rs11091748, rs11091748-rs17328236, rs1934179-rs4554617, rs1934183-rs1934179-rs4554617 and rs12171755-rs1934183-rs1934179-rs4554617 were statistically significant, even after the permutation test correction.

Conclusion: Our study provides strong evidence of an association between *DGKK* nucleotide variants, haplotypes and hypospadias susceptibility.

Keywords: *DGKK*; diacylglycerol kinase kappa; haplotypes; hypospadias; polymorphism.

INTRODUCTION

In hypospadias, the external urethral opening is positioned abnormally between the glans and the perineum, thus allowing the classification of hypospadias as anterior (distal), middle (midshaft) and posterior (proximal). Anterior hypospadias is described as glandular (the meatus on the ventral surface of glans penis), coronal, or subcoronal. In middle hypospadias the urethra opens into ventral surface of penis. In posterior hypospadias the urethral opening is located in the penoscrotal junction, scrotum, or perineum[1]. The majority of cases are isolated, i.e. individuals are not affected by other congenital anomalies. Hypospadias is the second most common human birth defect with an incidence of 1 in 250 live male births and its pathogenesis is complex, multifactorial, and determined by genetic, endocrine, and environmental causes[1-5]. Previous studies demonstrated familial reoccurrence for the anterior and middle forms of those malformations but not for posterior types, displaying the importance of genetic predisposition for hypospadias[5]. Many linkage analyses, aiming to elucidate the molecular genetic basis of hypospadias were performed in the past, but they have met with only limited success. In part, this limited success can be attributed to the complexity of the disease, as well as to the selection of not homogenous populations for investigations[4]. Recently, two genome-wide association studies based on DNA samples from West European cases[5,6], as well as two case-control studies conducted in the California population composed primarily of Hispanic and Caucasian individuals[7] and in the Han Chinese population[8], showed that common polymorphic variants of the *DGKK* gene can increase the risk of hypospadias.

The *DGKK* gene (OMIM *300837) located on chromosome Xp11.22 encodes the diacylglycerol kinase kappa. This enzyme is involved in the down-regulation of diacylglycerol signalling since it phosphorylates diacylglycerol, converting it to phosphatidic acid[9]. Determination of the exact associations between polymorphic variants of candidate genes and hypospadias risk might provide very important insight into the cause of hypospadias[4,10-12]. Expression of *DGKK* in preputial tissue is lower in boys with the hypospadias risk allele of rs1934179[5]. Recently, Shen et al.[13] reported that the enzyme *Dgkk* appears to be a mediator during development of mouse external genitalia.

The global burden incurred from hypospadias in terms of physical morbidity, health care expenses, emotional

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Table 1. Characteristics of polymorphisms genotyped in the *DGKK* gene.

Gene	rs no.	Location[a]	Alleles[b]	SNP function[c]	Protein effect	MAF[d]
DGKK	rs4074320	chrX:50119085	A / G (REV)	missense	p.Asp1118Asn	0.29
	rs4143304	chrX:50146570	C / T (FWD)	cds-synon	p.Leu368Leu	0.39
	rs11091748	chrX:50157984	A / G (FWD)	intron		0.39
	rs17328236	chrX:50168209	A / G (FWD)	intron		0.28
	rs12171755	chrX:50179749	C / T (FWD)	intron		0.35
	rs1934183	chrX:50181014	G / T (REV)	intron		0.39
	rs1934179	chrX:50182184	C / T (REV)	intron		0.36
	rs4554617	chrX:50203402	A / C (FWD)	intron		0.37
	rs4826634	chrX:50208239	G / T (FWD)	intron		0.37
	rs7063116	chrX:50235002	A / G (FWD)	N/A (upstream)		0.37

[a]NCBI build 37 / hg19.
[b]Underline denotes the minor allele (based on whole sample).
[c]According to the Single Nucleotide Polymorphism database (dbSNP)
[d]MAF, minor allele frequency calculated from the control samples
Abbreviations: FWD, forward; REV, reverse strand.

distress, and social dysfunction is significant for affected individuals, their families, and the health care system overall[2,11,14]. Hypospadiology remains a constantly evolving discipline with plenty of discrepancies among epidemiologic studies[1,11]. Identifying the underlying aetiology of this condition is crucial for improving prevention strategies and genetic risk counselling. The primary aim of our case-control study was to investigate the contribution of previously reported cases of polymorphic variants of the candidate *DGKK* gene to the incidence of hypospadias in a homogenous Polish population. This study is the first to represent patients with hypospadias of East European origin as part of a replicate sample to the previously described studies. The secondary aim was to test the association between common *DGKK* haplotypes and hypospadias susceptibility using different risk models.

METHODS

Patients and controls

Considering apparent etiologic heterogeneity of hypospadias, only isolated anterior and middle cases were included in the current case-control study[5]. A total of 166 unrelated boys (13 months to 10 years old) presenting with non-syndromic hypospadias and 285 unrelated healthy boys (13 months to 10 years old) with no family history of hypospadias or other structural anomalies were recruited from the Institute of Mother and Child in Warsaw. The control group was matched by age and place of birth. Case eligibility to the study was ascertained using the detailed medical records of each patient. The non-syndromic designation was based on diagnosis of isolated hypospadias with no other apparent cognitive and structural anomalies. Of the 166 boys ultimately enrolled, there were 110 (66%) anterior and 66 (34%) middle forms of hypospadias. The ancestry contributions were estimated to be 100% of Caucasian, Polish descent in both the hypospadias cases and the control group. Samples were obtained between January 2013 and June 2015. DNA was isolated from peripheral blood lymphocytes using the salting-out extraction procedure. The study was approved by the local Ethics Committee. Written and oral consent was obtained from the legal guardians of all the participants.

Single nucleotide polymorphism selection and genotyping

Single nucleotide polymorphisms (SNPs) are defined as loci with alleles that differ at a single base, with the rarer allele having a frequency of at least 1% in a random set of individuals in a population[15,16]. Ten SNPs in *DGKK* gene, previously detected to be associated with hypospadias[5-8], were evaluated in this study (**Table 1**).

Table 2. High-resolution melting curve analysis (HRM) conditions for the identification of polymorphisms genotyped in the data set .

Gene	rs no.	Alleles[2]	Primers for PCR amplification (5' – 3')	Annealing temp. (°C)	PCR product length (bp)	Melt. temp. range (°C)
*DGKK*1	rs4074320	A / G	F: GGGAATACAGGAAGCTGCAC R: ACCTGAGCAAGATCCACCAG	55	128	80 - 95
	rs4143304	C / T	F: TGCAGTCTTTGCTTGCTCTC R: TCACCAGATTCACACCCATC	55	96	78 - 93
	rs11091748	A / G	F: ACCCTACAGGACTGGACCATAG R: GAGACAGCCTTGTCACCTAGAAC	58	147	80 - 95
	rs17328236	A / G	F: TCACCACATCAAGGCTCTACC R: GCCACCCAATGGTGAATG	55	62	70 - 85
	rs12171755	C / T	F: GGGGTAGGCCAGGTAAGTAATG R: GGAAGTCAGAAGGCCAGAACA	58	122	75 - 90
	rs1934183	G / T	F: CTGGGAAGAGGCAGTAGTGG R: GTTCTTCTCCCCCACAGGA	61	135	80 - 95
	rs1934179	C / T	F: CATTTTTCTATCAATTGGCTCCT R: TCCAAATCTACACTCCTTTTTGC	55	136	75 - 90
	rs4554617	A / C	F: TTCATTCCCCTCTACTCTTGGA R: CCCTCAAGCACGTGTAGGAT	61	149	80 - 95
	rs4826634	G / T	F: CCATGGGCTTTGATGAGG R: GGACAGTGACCCCAGATAATG	58	111	74 - 89
	rs7063116	A / G	F: TGGACCTTGGTTGTTGATG R: CACAGTTGAAATCTGTTTTAGGAAC	55	169	71 - 86

[1]Genomic DNA for molecular analyses was isolated from peripheral blood lymphocytes by a standard salt-out extraction procedure.
[2]Underline denotes the minor allele (based on whole sample).

Table 3. Association of *DGKK* gene SNPs with the risk of hypospadias.

rs no.	Alleles[a]	Allele counts in cases[b]	MAF in cases	Allele counts in controls[b]	MAF in controls	OR (95% CI)[c]	*p* value
rs4074320	A / G	63 / 103	0.38 (A)	82 / 196	0.29 (A)	1.46 (0.97 - 2.19)	.0660
rs4143304	C / T	85 / 79	0.48 (C)	108 / 171	0.39 (T)	1.70 (1.15 - 2.52)	.0072
rs11091748	A / G	89 / 76	0.46 (A)	109 / 174	0.39 (G)	1.87 (1.27 - 2.76)	**.0015**
rs17328236	A / G	63 / 99	0.39 (G)	78 / 201	0.28 (G)	1.64 (1.09 - 2.47)	.0176
rs12171755	C / T	79 / 82	0.49 (T)	98 / 182	0.35 (T)	1.79 (1.21 - 2.66)	**.0037**
rs1934183	G / T	86 / 78	0.48 (T)	112 / 173	0.39 (G)	1.70 (1.15 - 2.51)	.0069
rs1934179	C / T	79 / 86	0.48 (T)	102 / 178	0.36 (T)	1.60 (1.08 - 2.37)	.0175
rs4554617	A / C	77 / 81	0.49 (C)	103 / 176	0.37 (C)	1.63 (1.10 - 2.43)	.0147
rs4826634	G / T	50 / 115	0.30 (T)	106 / 178	0.37 (T)	0.73 (0.48 - 1.10)	.1320
rs7063116	A / G	73 / 91	0.45 (A)	105 / 180	0.37 (A)	1.37 (0.93 - 2.03)	.1096

Statistically significant results (p-value < 0.005) are highlighted in bold font.
[a]Underline denotes the risk allele.
[b]The order of alleles d / D (d is the minor allele in the control samples).
[c]Allelic model: d vs D (d is the risk allele).
Abbreviations: MAF, minor allele frequency; OR, Odds Ratio; CI, confidence interval.

The genotyping was carried out by high-resolution melting curve analysis (HRM) on the LightCycler 480 system (**Table 2**). For quality control, approximately 10% of randomly selected samples were re-genotyped. Samples that failed genotyping were not repeated and were removed from statistical calculations.

Statistical methods

For each SNP, the Hardy-Weinberg (HW) equilibrium was evaluated in both patients and controls using Chi-square (χ^2) test. Statistically significant deviation from HW expectations was interpreted as p-value < .05. The differences in allele frequencies between cases and controls were determined using standard χ^2 test. The strength of association was estimated by Odds Ratio (OR) and corresponding 95% confidence intervals (95% CIs). The Bonferroni correction was applied to account for multiple comparisons, and p-values < .005 (.05 / 10 SNPs) were interpreted as statistically significant.

The haplotype-based association analysis was performed using PLINK v1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/). The omnibus haplotype test (jointly estimating all haplotype effects at a given location) for sliding windows of 2 to 4 SNPs across the gene was conducted using logistic regression. Significant p-values were corrected using the 1,000-fold permutation test. The detailed haplotype analysis was conducted for SNP combinations with statistically significant Omnibus test p-values. Haplotype-specific odds ratios (ORs) were calculated and the most common haplotypes were used as the reference. Only haplotypes with frequencies ≥ 0.01 in either cases or controls were tested.

RESULTS

First, we analyzed the *DGKK* SNPs independently. None of the tested SNPs showed evidence of deviation from Hardy-Weinberg equilibrium in neither the cases nor the controls. After correction for multiple testing, statistically significant results of increased risk for hypospadias were observed only for carriers of the *DGKK* rs11091748 and rs12171755 variants (**Table 3**). The OR for individuals with the rs11091748 G allele compared to A allele carriers was 1.87 (95% CI = 1.27 - 2.76, P = .0015). Six other SNPs showed a trend toward association with hypospadias. The *DGKK* nucleotide variants demonstrated moderate linkage disequilibrium (LD). D' and r^2 values, calculated from the genotype data of the control samples, ranged from 0.607 to 1.000 and 0.131 to 0.984, respectively (Figure 1 and Table 4). Subsequently, we tested the common *DGKK* haplotypes for their association with the risk of hypospadias. The global p-values for the two two-markers haplotypes (rs11091748_rs17328236, rs1934179_rs4554617), the one three-markers haplotype (rs1934183_rs1934179_rs4554617), and the one four-markers haplotype (rs12171755_rs1934183_rs1934179_rs4554617) were statistically significant even after permutation correction. Detailed analysis of those haplotypes is presented in Table 5. All tested SNPs were involved in haplotype combinations associated with hypospadias. However, the haplotype combination (rs1934179_rs4554617) with the best global p-value (pcorr = .007, Table 5) does not include the two SNPs (rs11091748 and rs12171755) highly linked with hypospadias in the single markers analysis (**Table 3**).

Table 4. Linkage disequilibrium between markers of the *DGKK* gene in the control samples.

	rs4074320	rs4143304	rs11091748	rs17328236	rs12171755	rs1934183	rs1934179	rs4554617	rs4826634	rs7063116
rs4074320		0.861	0.849	0.963	0.757	0.766	0.752	0.750	0.746	0.607
rs4143304	0.489		1.000	1.000	0.983	0.889	0.984	0.952	0.891	0.763
rs11091748	0.469	0.984		1.000	0.982	0.869	0.983	0.948	0.917	0.757
rs17328236	0.850	0.604	0.594		0.882	0.889	0.879	0.878	0.962	0.730
rs12171755	0.448	0.816	0.792	0.558		0.982	1.000	0.984	0.909	0.818
rs1934183	0.372	0.777	0.756	0.468	0.796		0.983	0.966	0.892	0.758
rs1934179	0.415	0.870	0.848	0.521	0.940	0.851		0.984	0.913	0.774
rs4554617	0.406	0.827	0.802	0.512	0.896	0.835	0.954		0.942	0.805
rs4826634	0.131	0.283	0.300	0.203	0.247	0.295	0.266	0.291		0.740
rs7063116	0.276	0.513	0.503	0.366	0.638	0.498	0.590	0.627	0.178	

D' above diagonal
r^2 below diagonal

Table 5. Haplotype analysis of SNPs genotyped in the *DGKK* gene.

Polymorphisms	*p*-value	Omnibus haplotype test Corrected *p*-value[a]	Haplotype	Frequency Cases	Controls	Odds Ratio[b]	*p*-value[c]
2-marker window							
rs4074320_rs4143304	.0561	.2777					
rs4143304_rs11091748	.0017	.0150	C-A	0.466	0.620	Referent	
			T-G	0.522	0.376	1.84 (1.39 - 2.44)	< 0.0001
			C-G	0.012	0.004	4.29 (0.78 - 23.68)	.0883d
rs11091748_rs17328236	.0084	.0440	A-A	0.466	0.616	Referent	
			G-G	0.391	0.270	1.92 (1.41 - 2.61)	< 0.0001
			G-A	0.143	0.114	1.66 (1.08 - 2.55)	.0208
rs17328236_rs12171755	.0134	.0719					
rs12171755_rs1934183	.0210	.1089					
rs1934183_rs1934179 .0142	.0749						
rs1934179_rs4554617 .0004	.0070		C-A	0.459	0.627	Referent	
			T-C	0.446	0.362	1.68 (1.26 - 2.25)	.0004
			C-C	0.044	0.007	8.507 (2.75 - 26.29)	< 0.0001[d]
			T-A	0.051	0.004	19.444 (4.41 - 85.68)	< 0.0001[d]
rs4554617_rs4826634	.0502	.2418					
rs4826634_rs7063116	.0568	.2867					
3-marker window							
rs4074320_rs4143304_rs11091748	.0241	.1149					
rs4143304_rs11091748_rs17328236	.0104	.0559					
rs11091748_rs17328236_rs12171755	.0130	.0709					
rs17328236_rs12171755_rs1934183	.0201	.1019					
rs12171755_rs1934183_rs1934179	.0290	.1469					
rs1934183_rs1934179_rs4554617	.0023	.0180	T-C-A	0.442	0.591	Referent	
			G-T-C	0.430	0.364	1.58 (1.17 - 2.13)	.0026
			G-C-A	0.019	0.030	0.85 (0.32 - 2.21)	.7357
			G-T-A	0.051	0.004	18.09 (4.10 - 79.77)	< 0.0001[d]
			G-C-C	0.039	0.004	13.56 (2.99 - 61.45)	< 0.0001[d]
			T-T-C	0.013	0.004	4.52 (0.82 0 24.99)	.0785d
rs1934179_rs4554617_rs4826634	.0397	.1928					
rs4554617_rs4826634_rs7063116	.1930	.7243					
4-marker window							
rs4074320_rs4143304_rs11091748_rs17328236	.0394	.1928					
rs4143304_rs11091748_rs17328236_rs12171755	.0187	.0959					
rs11091748_rs17328236_rs12171755_rs1934183	.0741	.3526					
rs17328236_rs12171755_rs1934183_rs1934179	.0377	.1808					
rs12171755_rs1934183_rs1934179_rs4554617	.0076	.0430	C-T-C-A	0.438	0.591	Referent	
			T-G-T-C	0.431	0.349	1.67 (1.23 - 2.26)	.0008
			C-G-C-A	0.020	0.030	0.87 (0.33 - 2.28)	.7816
			C-G-T-C	0.007	0.015	0.58 (0.12 - 2.78)	.7302d
			T-G-T-A	0.046	0.004	16.30 (3.65 - 72.73)	< 0.0001[d]
			C-G-C-C	0.013	0.004	4.66 (0.84 - 25.74)	.0734[d]
			T-T-T-C	0.013	0.004	4.66 (0.84 - 25.74)	.0734[d]
			T-G-C-C	0.020	0.000	20.91 (1.12 - 391.39)	.0086[d]
rs1934183_rs1934179_rs4554617_rs4826634	.0985	.4555					
rs1934179_rs4554617_rs4826634_rs7063116	.0509	.2428					

Detailed haplotype analysis was presented only for SNP combinations with statistically significant Omnibus test *p*-values.
[a]*p*-value calculated using permutation test and a total of 1,000 permutations.
[b]The most common haplotype was used as the reference.
[c]Chi-square test.
[d]Fisher exact test.

DISCUSSION

Identifying the major genetic alternations leading to hypospadias will have an impact on genetic counselling and will lead to a greater understanding of the male urinary tract development. Our study builds on previous publications which have reported that the genetic susceptibility of hypospadias may be associated with common variants of the *DGKK* gene[5-8]. In our mono-ethnic sample, the *DGKK* haplotypes were found to be strongly associated with hypospadias and provided further evidence that *DGKK* may be an important disease-promoting gene[10,11,15,16]. The high odds ratios and level of significance provide compelling support for the observed haplotypes associations, despite the small numbers of participants. For the two investigated SNPs (rs4826634 and rs7063116), in the presented Polish sample of patients, evidence of association with hypospadias was found only using haplotypes testing. The lack of association in the single marker analysis may be attributed to a lack of power, secondary to small sample size. An alternative explanation might be that the analyzed variants do not target the causal variant in the Polish population adequately, due to the presence of differing haplotypic structures in specific mono-ethnic populations[4,10,15]. In accordance with our study, Carmichael et al.[7] have previously found evidence of association between two blocks of *DGKK* haplotypes and the hypospadias risk in Californian population. In their study, an 8-SNPs block contained rs12171755, rs19341179 and rs19341179, which were also associated with increased risk of being born with hypospadias in the Polish population. In contrast to our results, Ma et al.[8] did not observe the association between *DGKK* haplotypes and hypospadias susceptibility in the Han Chinese population. However, a very recent study by Xie et al.[17] from China, similarly to our results, showed strong association of haplotypes including rs4554617 with the susceptibility to hypospadias. These findings support the assumption that the functional variants associated with these risky SNPs of *DGKK* are likely to be regulatory in nature. More in-depth investigations are necessary to explore the functional and mechanistic

role of *DGKK* in the male urinary system. Rigorously establishing the genetic risk for any multifactorial disorder is important but inherently difficult[4,10].

CONCLUSIONS

Our study represents a step forward in understanding the genetic basis of isolated hypospadias. The study provides strong evidence of an association of *DGKK* haplotypes with the susceptibility to hypospadias. Further testing in independent populations and meta-analyses are needed to clarify the role of nominally significant polymorphic variants of the *DGKK* gene association with hypospadias.

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CONFLICT OF INTEREST

The authors state that there are no conflicts of interest regarding the publication of this article.

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